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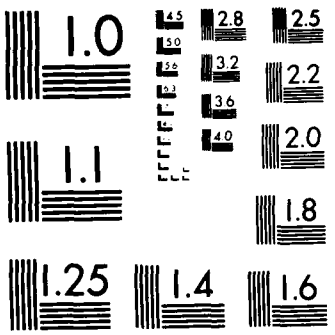

SECOND

ANNUAL REPORT

"Synthesis of a Marine Bioadhesive Protein"

Contract NR# N00014-84-K-029

by

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19. ABSTRACT (*Continue on reverse if necessary and identify by block number*)

In an attempt to synthesize analogs of the mussel adhesive protein (i.e (A-K-P-S-Y-Hyp-Hyp-T-Dopa-K)$_{80}$) using recombinant DNA and enzymatic technologies, we were faced two major challenges: 1) How to prevent extensive recombination of inserted highly repeated gene sequences in E. coli and Saccharomyces cerevisiae, and 2) How to bring about efficient enzymatic hydroxylation of tyrosyl and prolyl residues in expressed analogs. Researchers at Genex Corporation have succeeded in cloning and expressing translation of mussel adhesive analogs. These contain essentially 20 tandem repeats of the decapeptide excepting 3 T-P-A linkers. Peptide tyrosyl hydroxylation was achieved using mushroom polyphenoloxidase in the presence of ascorbate (BBA (1986) 872, 98). Overall conversion of tyrosine to dopa in the 20-mer was 40-50%. Prolyl hydroxylase has been partially purified from mussel tissue and characterized with respect to substrate specificity.

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I. Summary

In an attempt to synthesize analogs of the mussel adhesive protein (i.e (A-K-P-S-Y-Hyp-Hyp-T-Dopa-K)$_{80}$) using recombinant DNA and enzymatic technologies, we faced two major challenges: 1) How to prevent extensive recombination of inserted highly repeated gene sequences in E. coli and Saccharomyces cerevisiae, and 2) How to bring about efficient enzymatic hydroxylation of tyrosyl and prolyl residues in expressed analogs. Researchers at Genex Corporation have succeeded in cloning and expressing translation of mussel adhesive analogs. These contain essentially 20 tandem repeats of the decapeptide excepting 3 T-P-A linkers. Peptide tyrosyl hydroxylation was achieved using mushroom polyphenoloxidase in the presence of ascorbate (BBA (1986) 872, 98). Overall conversion of tyrosine to dopa in the 20-mer was 40-50%. Prolyl hydroxylase has been partially purified from mussel tissue and characterized with respect to substrate specificity.

II. Research Results

A. Experimental Approach

The preparation of 20-mer from E. coli and S. cerevisiae is described in the following section by Genex Corporation. Hydroxylation of tyrosines in the 20-mer was performed at 20°C with constant aeration and stirring. Usually 6 mg of the 20-mer and 250 µmole ascorbic acid were incubated with 0.5 mg polyphenoloxidase (Sigma) in 10 ml 0.1 M phosphate buffer pH 6.8. Enzyme activity was assayed on alternate days following tyrosine to dopa conversion at 280 nm to determine to what degree aging led to a loss of activity (Worthington, 1972). The reaction was continued for 3 hrs to achieve a nearly asymptotic end point (see BBA (1986) 872, 98). At this state the pH was readjusted to 8.0 with 0.1 M NaOH and phosphate buffer to 0.05 M with distilled water. The sample was then applied to a boronate affinity column (Pierce Glycogel B 3.2 cm x 1 cm) previously equilibrated with 0.05 M phosphate pH 8.0. Non-dopa containing proteins were eluted with 50-100 ml phosphate buffer; dopa peptides were washed off with 5% acetic acid (Hawkins et al, 1986). Dopa-rich fractions were detected by the nitrite-molybdate reaction and then pooled and resolved by reversed phase HPLC on C-8 silica (Brownlee, RP-300) using an acetonitrile gradient in water and 0.1% trifluoroacetic acid (Waite et al., 1985). Solvent was removed by freeze-drying resulting in a dopa-rich protein fluff. Best yields at this stage from the initial unmodified 20-mer were 30%.

For further characterization the freeze-dried dopa containing 20-mer was dissolved in 3 ml 0.05 M sodium borate (pH 8.0) containing 0.001 M $CaCl_2$. Trypsin (TPK inhibited) was added at an enzyme to protein ratio of 1:100 and incubated at 25°C for 12 h and 25 psi N_2 (Waite et al., 1985). The sample was then flash evaporated at 40°C to a final volume of 1 ml and applied to a column of LH-Sephadex 20 (1.5 cm x 75 cm) eluted with 0.2 M acetic acid. Peak fractions with dopa were again collected, flash evaporated and separated using a shallow acetonitrile gradient (9-30%) on C-8 silica (Brownlee RP-300). Hydrolysis followed by amino acid analysis was routinely performed on purified dopa-peptides.

B. Results

High tyrosine-to-dopa conversions using mushroom polyphenoloxidase are achievable using polyphenoloxidase on the 20-mer. Pre- and post-hydroxylation 20-mer was routinely evaluated by SDS gel electrophoresis, acid-urea gel electrophoresis (Waite and Benedict, 1984), reversed phase HPLC and amino acid analysis. Upon hydroxylation the 20-mer (dimer, initial M_W 30,000) fails to be detectable in SDS gels following electrophoresis (Fig. 1). This is probably due to precipitation of SDS or protein insolubility in this buffer because the acid-urea gel system, protein- and dopa-staining bands are visible before and after hydroxylation (Fig. 2).

Retention of the pre- and post-hydroxylated 20-mer on C-8 HPLC columns is demonstrated in Fig. 3A & B. Note the sharpness

of the prehydroxylated 20-mer in Fig. 3A and the broader more diffuse and faster eluting pattern for the hydroxylated analog. Amino acid composition of the two proteins is shown in Table 1. Note that the dopa to tyrosine ratio varies depending on the relative position in the peak.

Tryptic peptides of the hydroxylated 20-mer were prepared to determine whether mushroom polyphenoloxidase showed the same preference as it did for Tyr-9 in the decapeptide (Marumo and Waite, 1986). Fig. 4 illustrates the elution profile for tryptic peptides from the 20-mer. Curiously, although the peptides elute at slightly shorter retension times than the present 20-mer, they are much more strongly retained then tryptic decapeptides from natural mussel adhesive protein. This difference may be due to the presence of hydroxyproline in the latter. Most of the tryptically derived decapeptides from the mussel adhesive protein eluted between 9-11% acetonitrile whereas synthetic decpeptides were retained on C-8 until about 20% acetonitrile. Amino acid compositions of 20-mer tryptic peptides are shown in Table II. Note that the dopa/tyr ratios in the leading peak run as high as 3 to 1 whereas in the trailing peak the ratio is closer to 1:1. Sequences of these peptides are presently being determined.

Hawkins, C.J., Lavin, M. F., Parry, D. L. and Ross, I. L. (1986). Analyt. Biochem. 159, in press.

Marumo, K. and Waite, J. H. (1986). Biochim. Biophys. Acta 872: 98-104.

Waite, J. H. and Benedict, C.V. 1(984). Methods Enzymol. 107, 397-412.

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Worthington Enzyme Manual (1972). Worthington Biochemical Corporation, Freehold, NJ.

TABLE I. Amino acid composition (res/1000) of mussel adhesive 20-mer analogs.

AMINO ACID	NONHYDROXYLATED 20-MER	HYDROXYLATED 20-MER	HYDROXYLATED HPLC 1	2	3	5	7	9	11
THR	97	99	90	97	92	106	105	106	103
SER	90	96	98	94	98	90	85	83	85
PRO	302	290	287	325	302	340	334	337	329
ALA	129	123	102	114	117	128	127	124	123
DOPA	0	56	79	116	121	77	86	82	81
TYR	146	77	11	11	14	53	62	65	75
LYS	150	139	159	164	150	171	161	157	153
$\frac{\text{DOPA}}{\text{DOPA+TYR}} \times 100 =$	0	42	88	91	90	59	58	56	52

TABLE II. Amino acid composition (res/1000) of peptides derived from hydroxylated 20-mer by trypsin digestion. Fractions listed are are the same as those designated as such under the profile in Figure 4.

AMINO ACID	20-MER TRYPTIC DIGEST HPLC FRACTIONS					
	1	2	3	4	5	6
THR	118	107	103	94	106	108
SER	113	114	106	140	105	104
PRO	330	304	300	255	301	309
ALA	99	94	95	96	105	106
DOPA	119	119	121	101	94	95
TYR	37	67	72	72	97	99
LYS	155	168	170	161	163	162
$\frac{\text{DOPA}}{\text{DOPA+TYR}} \times 100 =$	76	64	63	58	49	49

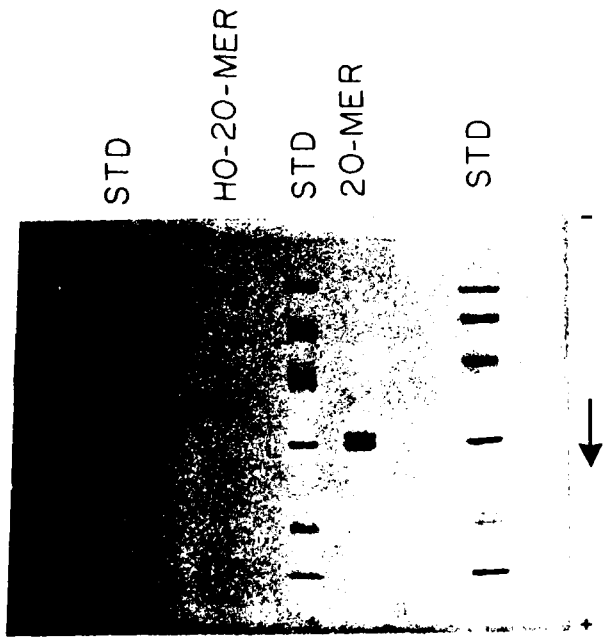
FIGURE 1. Sodium dodecylsulfate polyacrylamide gel electrophoresis. The mixture of standard proteins in lanes 1, 4, and 7 consists of phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), respectively at 5 ug each. Lanes for hydroxylated and prehydroxylated 20-mer (10 ug each) are 3 and 5, respectively. Protein stain is Serva Blue R-250.

FIGURE 2. Polyacrylamide (7.5%) gel electrophoresis in 5 % acetic acid and 2 M urea. Applied 10 ug each of trypsinized hydroxylated 20-mer, hydroxylated 20-mer and prehydroxylated 20-mer. The trypsinized material did not fix for staining but can be visualized by treatment with molybdate-nitrite and has a relative mobility of 0.73. Protein stain is Serva Blue R-250.

FIGURE 3. Reversed phase high performance liquid chromatography of the Genex 20-mer (S. cerevisiae) on C-8 silica. A. Prehydroxylated 20-mer; B. 20-mer hydroxylated by treatment with mushroom polyphenoloxidase.

FIGURE 4. Reversed phase high performance liquid chromatography of trypsin-digested hydroxylated 20-mer on C-8 silica.

FIG. 1



12 % PAGE with SDS

FIG. 2

5 % PAGE with ACETIC ACID/UREA

Fig 3

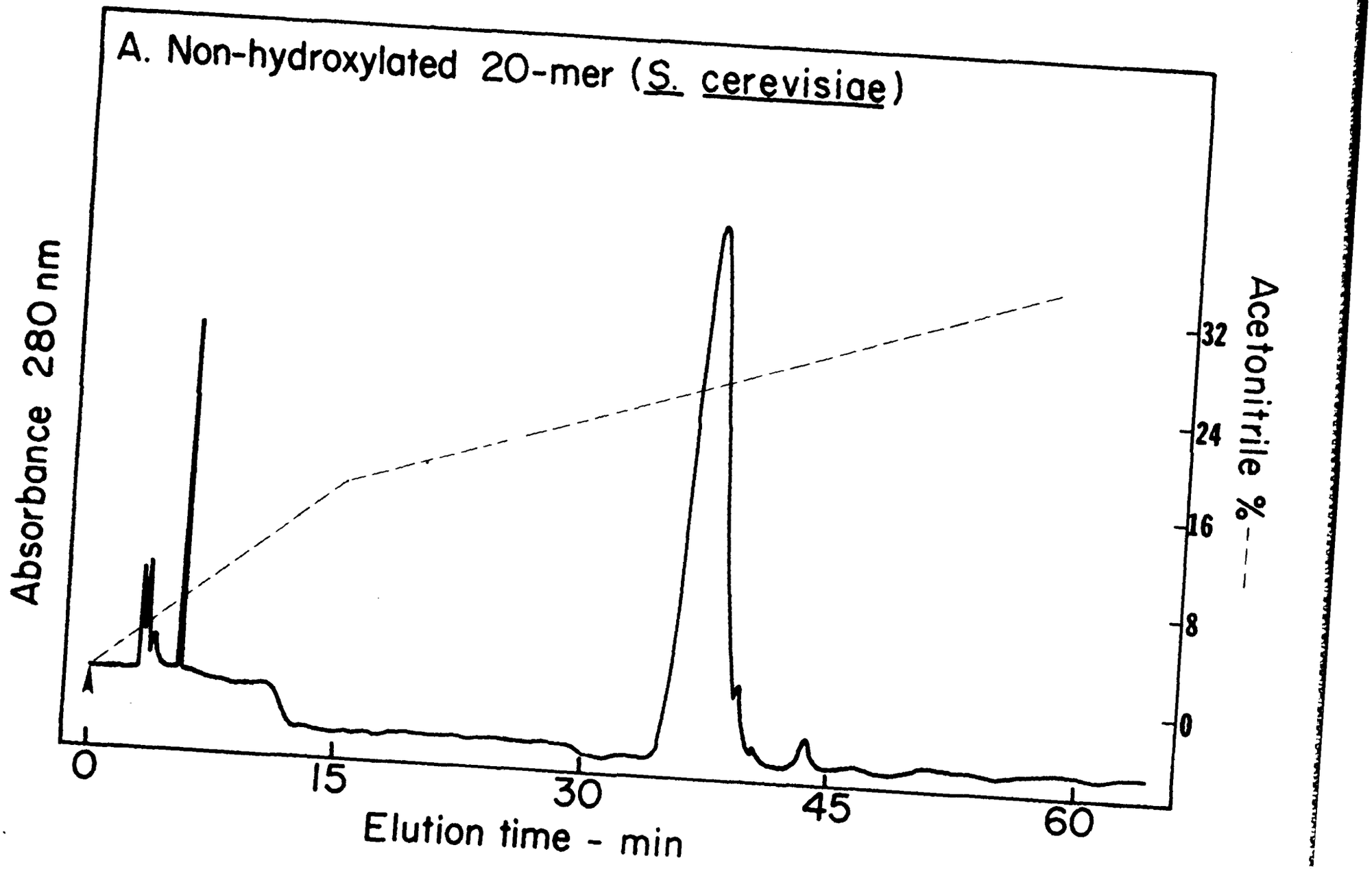

A. Non-hydroxylated 20-mer (S. cerevisiae)
Absorbance 280 nm
Acetonitrile %
32
24
16
8
0
0
15
30
45
60
Elution time - min

Fig 3

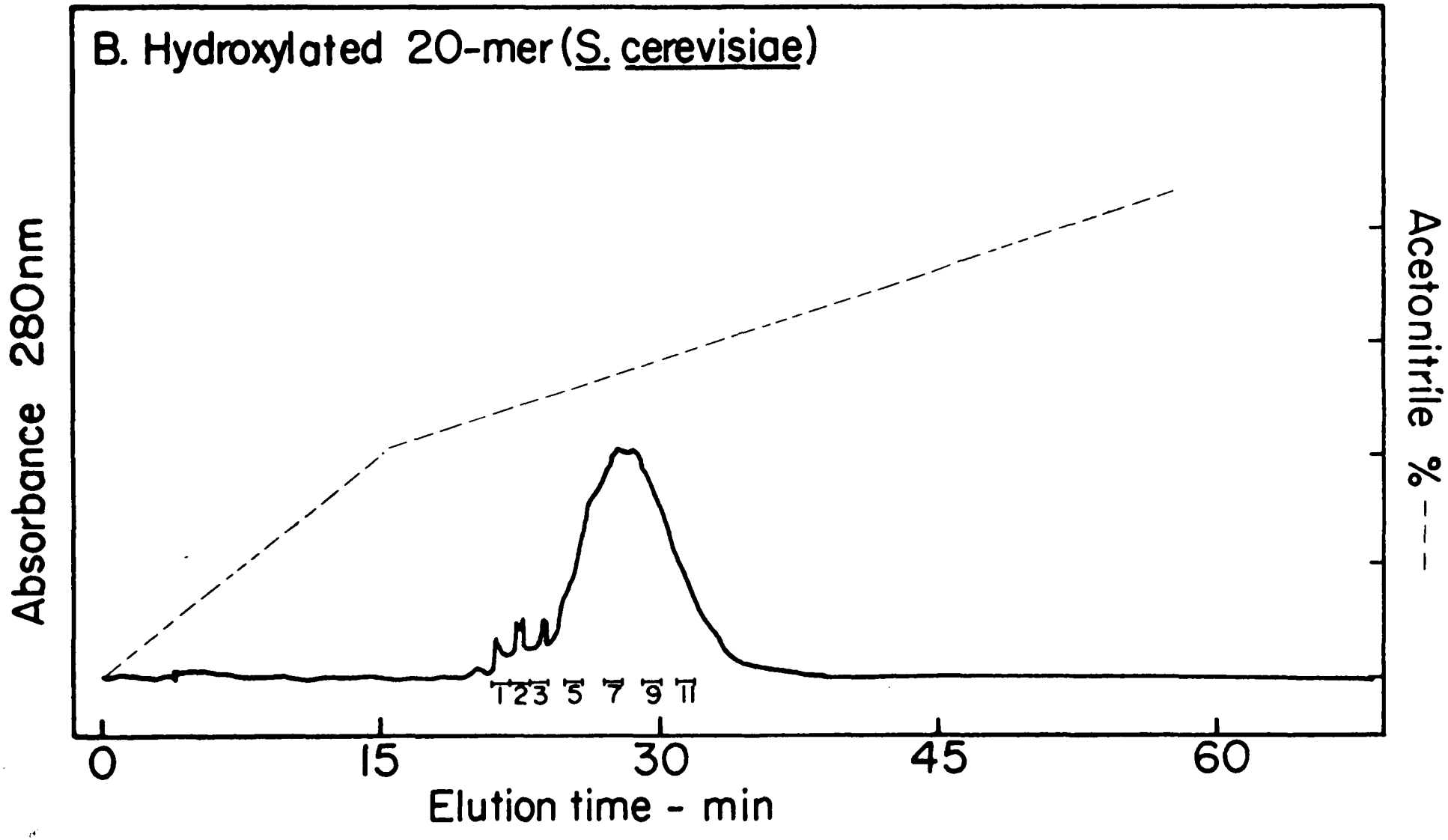
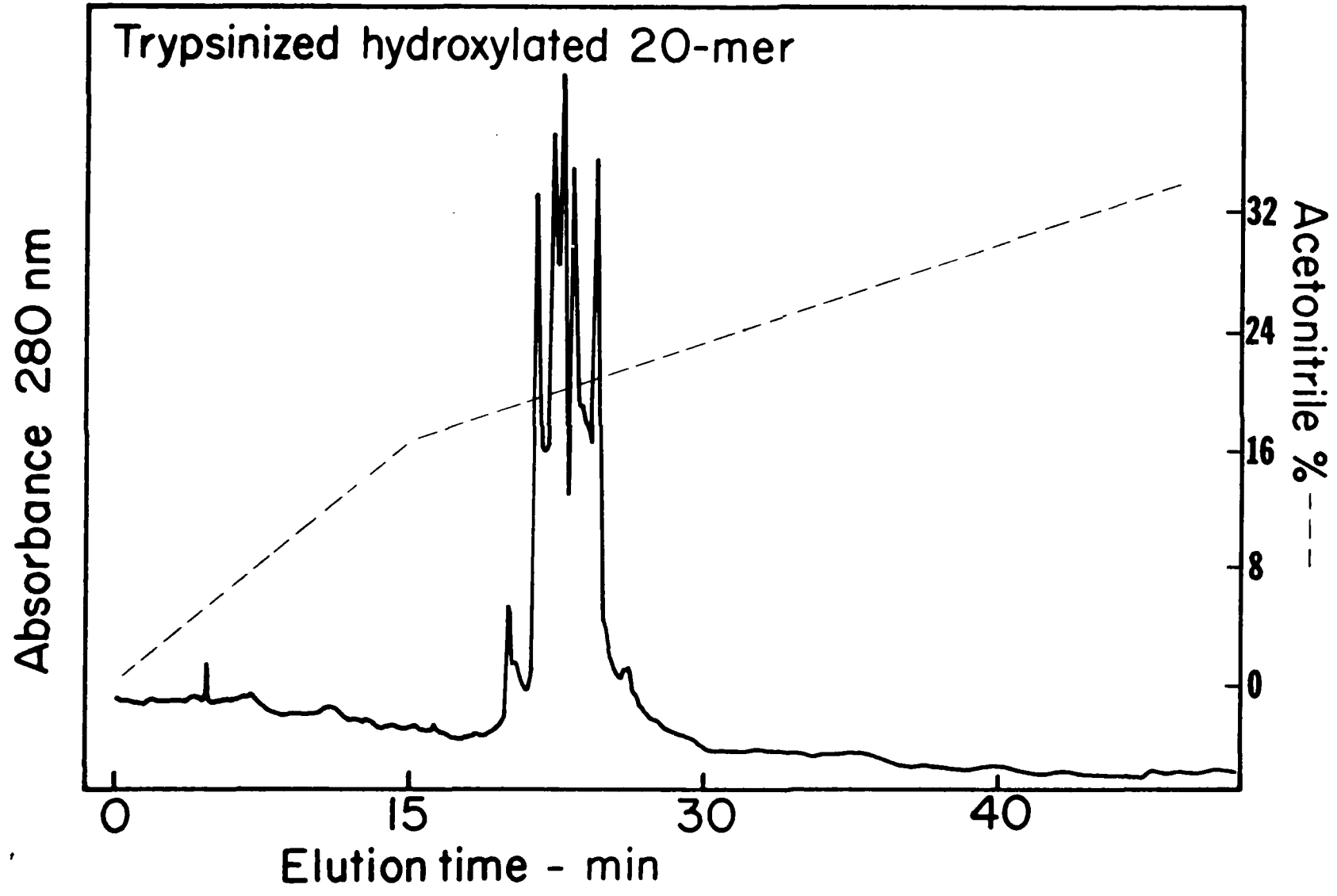

B. Hydroxylated 20-mer (S. cerevisiae)
Absorbance 280nm
Acetonitrile % ---
1 2 3 5 7 9 11
0
15
30
45
60
Elution time - min

Fig 4


Trypsinized hydroxylated 20-mer
Absorbance 280 nm
Acetonitrile % - - -
32
24
16
8
0
0
15
30
40
Elution time - min

END

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